Kinetic significance of $GroEL_{14}\cdot(GroES_7)_2$ complexes in molecular chaperone activity

Fernando J Corrales and Alan R Fersht

Background: Symmetrical $GroEL_{14}\cdot(GroES_7)_2$ complexes, nicknamed 'footballs', have been observed by electron microscopy to form in the presence of excess ATP. But the significance of these footballs in the molecular chaperone cycle is controversial. We have analyzed the folding of barnase in the presence of GroEL, GroES and various nucleotides to probe the importance of footballs.

Results: A stoichiometric concentration of $GroES_7$ binds to the $GroEL_{14}$·nucleotide·denatured barnase complex to produce a slow-folding state. Higher concentrations of GroES in the presence of ATP or AMP-PNP, but not ADP, produce a proportion of a fast-folding state, rising to 50% at a $GroES_7$: $GroEL_{14}$ stoichiometry of ≥2:1.

Conclusions: These results imply that there is a transiently formed $GroEL_{14}\cdot(GroES_7)_2$·denatured protein complex that dissociates into a 50:50 mixture of slow-folding *cis* and fast-folding *trans* $GroEL_{14}\cdot GroES_7$·denatured protein complexes. The transient formation of a symmetrical football could provide a means of opening the cage that encapsulates folded *cis*-bound proteins.

Address: MRC Unit for Protein Function and Design and Cambridge Centre for Protein Engineering, Department of Chemistry, University of Cambridge, Lensfield Road, Cambridge CB2 1EW, UK.

Correspondence: Alan R Fersht
e-mail: arf10@cam.ac.uk

Key words: barnase, chaperonin, football, protein folding

Received: 25 Mar 1996
Revisions requested: 25 Apr 1996
Revisions received: 09 May 1996
Accepted: 10 May 1996

Published: 19 Jun 1996
Electronic identifier: 1359-0278-001-00265

Folding & Design 19 Jun 1996, 1:265–273

Introduction

The folding of some proteins *in vivo* and *in vitro* is aided by molecular chaperones, such as GroEL in *Escherichia coli* [1,2]. GroEL is a typical member of the cpn60 class of chaperonins, which are 14-mers that consist of two stacked seven-membered rings with a large central cavity [3,4]. The co-chaperonin GroES [5] is a seven-membered ring that binds to the ends of the GroEL cylinder in the presence of ATP, ADP or non-hydrolyzable analogues such as AMP-PNP. Collapsed denatured states of proteins have been observed by electron microscopy to bind in the GroEL·GroES·nucleotide complex, in the ring of GroEL that is bound to GroES (i.e. GroES and the substrate are *cis*), and also in the opposite ring (i.e. *trans*) [6–8]. The binding of GroES causes the *cis* ring of GroEL to have a larger cavity than the *trans* [7]. Denatured proteins bind initially to the *trans* ring of the GroEL·GroES·nucleotide complex [9,10]. But GroES dissociates on the hydrolysis of ATP and then rebinds to the other end of the transiently formed GroEL·nucleotide·denatured protein complex so that the substrate ends up in the *cis* ring [9,10]. A proportion of GroEL in the presence of excess GroES and ATP forms a symmetrical $GroEL_{14}\cdot(GroES_7)_2$ complex, termed a 'football' [11–14]. The significance of footballs in the chaperone cycle is not clear and it has been argued that they are artefactual [15]; however, there are correlations between the presence of symmetric complexes in a reaction mixture and the activity in the folding of rubisco [13] and of rhodanese [14].

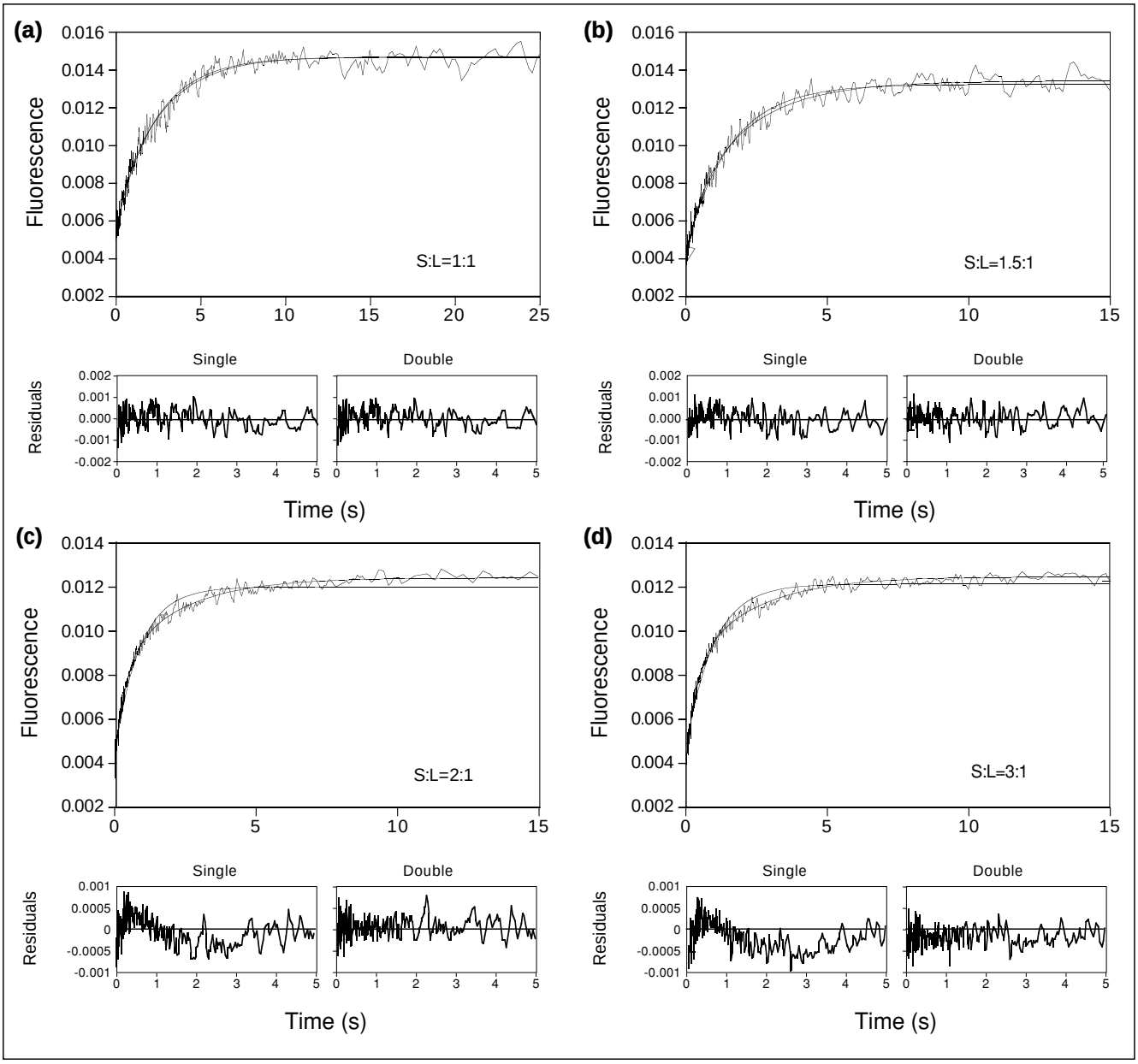
Individual steps in the mechanism of action of GroEL can be followed by mixing it with denatured barnase, GroES, and nucleotides in different combinations and permutations to produce different intermediate states in the chaperoning cycle [16]. Barnase is a 110-residue ribonuclease that does not contain cysteine crossbridges and whose folding pathway *in vitro* is known in considerable detail. GroEL acts as both a folding cage and an annealing machine for barnase: denatured barnase folds completely while bound to GroEL [17,18]; and GroEL catalyzes under physiological conditions the exchange with solvent of deeply buried NH protons of native barnase that are exposed only on full unfolding of the protein [19]. We have now performed experiments on the folding of denatured barnase in the presence of GroEL and GroES that test the role of footballs in molecular chaperone activity.

Results

Folding of barnase on the addition of GroES and nucleotides to preformed GroEL·denatured barnase complexes

Folding was initiated by mixing the mutant barnase Trp94→Tyr that had been denatured in 32 mM HCl (pH 1.5) with a folding buffer (containing various concentrations of GroEL or GroES and nucleotides, where appropriate) to give final concentrations of 100 mM 2-[*N*-morpholino]ethanesulfonic acid (MES), pH 6.3, 2 mM KCl, 2 mM $MgCl_2$, 1 μM barnase, and generally 2 μM $GroEL_{14}$, at 25°C.

Figure 1

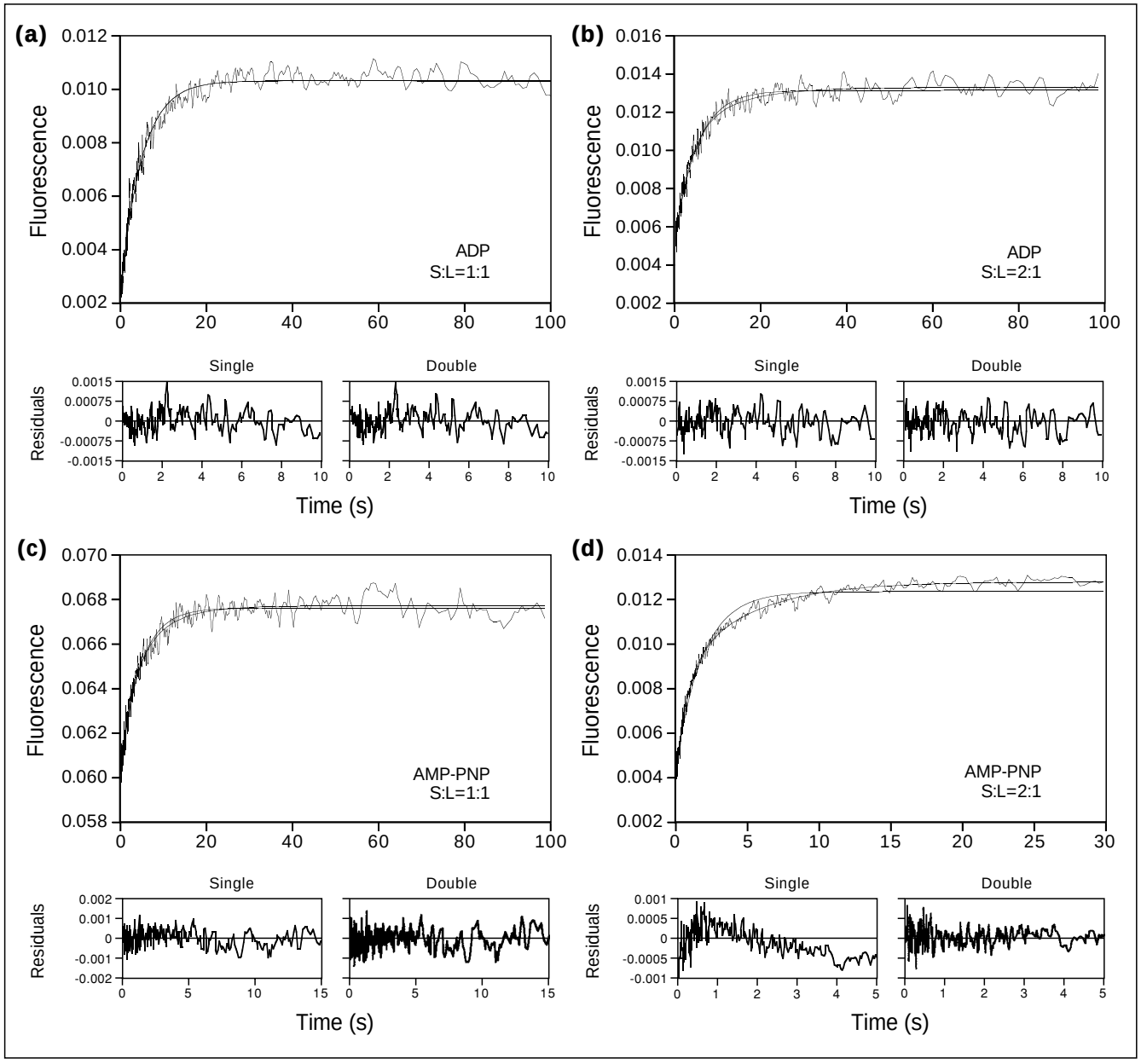


Folding of barnase on the addition of GroES and ATP to preformed GroEL·denatured barnase complexes. (a) $GroES_7 : GroEL_{14}$ = 1:1. (b) $GroES_7 : GroEL_{14}$ = 1.5:1. (c) $GroES_7 : GroEL_{14}$ = 2:1. (d) $GroES_7 : GroEL_{14}$ = 3:1.

Effects of ATP

In Figure 1, equal volumes of acid-denatured barnase (4 μM) and $GroEL_{14}$ (8 μM in folding buffer) were mixed for 50 ms (incubation for 1 s gave the same results). An equal volume of GroES (in 100 mM MES pH 6.3, containing 2 mM KCl, 2 mM $MgCl_2$ and 1 mM ATP) was added and the fluorescence was monitored. The fit to each curve was examined by visual inspection of the observed data and theoretical fits (solid lines), the differences between the observed data and those calculated from the different theoretical fits plotted (the residuals), and the statistical analysis of the fit (χ^2). The panels in Figure 1 are for different GroES : GroEL ratios, with the residuals plotted underneath. The small differences in beginning and end points result from slight differences in the offset of fluorescence (~0.02 units). In Figure 1a, $GroES_7 : GroEL_{14}$ = 1:1. There is an excellent fit to a single exponential, as seen by the random distribution of the residuals about zero. The rate constant is 0.430 ± 0.007 s^{-1}, the amplitude 0.00898 ±

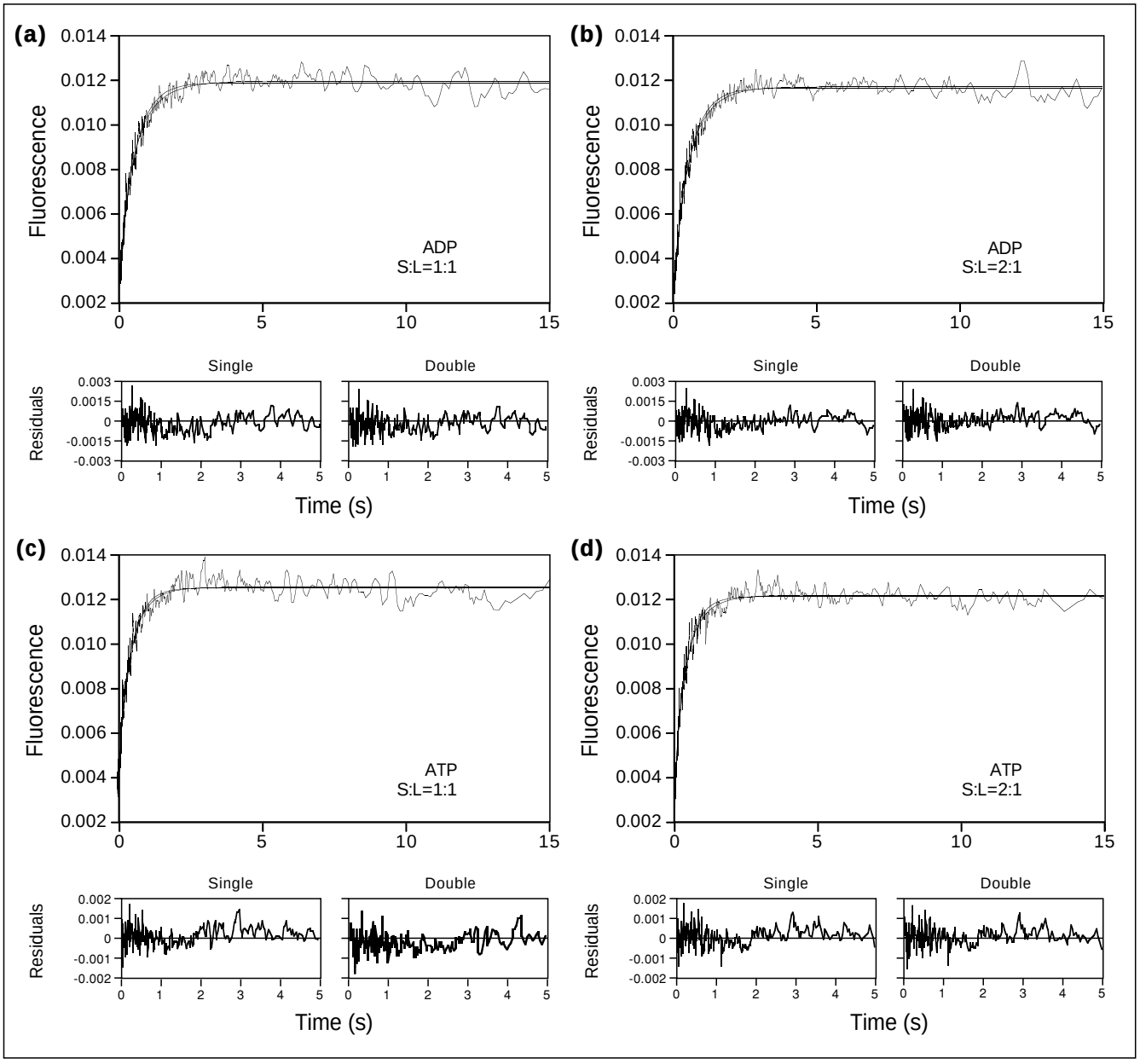
Figure 2



Folding of preformed GroEL-bound barnase on the addition of ADP or AMP-PNP and GroES. (a) 10 mM ADP at 1:1 GroES$_7$: GroEL$_{14}$. (b) 10 mM ADP at 2:1 GroES$_7$: GroEL$_{14}$. (c) 15 mM AMP-PNP at 1:1 GroES$_7$: GroEL$_{14}$. (d) 15 mM AMP-PNP at 2:1 GroES$_7$: GroEL$_{14}$.

0.00006 units and $\chi^2 = 6.2 \times 10^{-5}$. The fit to a double exponential has $\chi^2 = 1.05 \times 10^{-4}$. In Figure 1c, GroES$_7$: GroEL$_{14}$ = 2:1. The time course is clearly biphasic, as seen from the fit to the raw data. There is a random distribution of the residuals about zero for the fit to a double exponential, whereas the fit to the single exponential has the characteristic wavy deviation that is observed when significant additional phases are omitted from a fit. The rate constants for the double exponential are 2.65 ± 0.12 s^{-1} and 0.45 ± 0.02 s^{-1}; the respective amplitudes are 0.0043 ± 0.0002 units and 0.0041 ± 0.0002 units; and $\chi^2 = 2.95 \times 10^{-5}$, compared with $\chi^2 = 7.4 \times 10^{-5}$ for the single exponential. In Figure 1b, GroES$_7$: GroEL$_{14}$ = 1.5:1. A better fit is seen to the double exponential: there is a random distribution of the residuals about zero for the double exponential, and waves can just be detected in the residuals for the fit to the single exponential. This is reflected in $\chi^2 = 6.8 \times 10^{-5}$

Figure 3



Folding of barnase on addition to preformed GroEL·GroES·nucleotide complexes. (a) 10 mM ADP at 1:1 $GroES_7$: $GroEL_{14}$. (b) 10 mM ADP at 2:1 $GroES_7$: $GroEL_{14}$. (c) 1 mM ATP at 1:1 $GroES_7$: $GroEL_{14}$. (d) 1 mM ATP at 2:1 $GroES_7$: $GroEL_{14}$.

for the double exponential compared with $\chi^2 = 8.1 \times 10^{-5}$ for the single exponential. The rate constants for the double exponential are 1.9 ± 0.4 s^{-1} and 0.47 ± 0.03 s^{-1}; and the respective amplitudes are 0.0024 ± 0.0006 units and 0.0068 ± 0.0006 units. In Figure 1d, $GroES_7$: $GroEL_{14}$ = 3:1, the rate constants for the double exponential are 2.6 ± 0.1 s^{-1} and 0.44 ± 0.01 s^{-1}; the respective amplitudes are 0.0040 ± 0.0002 units and 0.0041 ± 0.0002 units; and $\chi^2 = 2.9 \times 10^{-5}$.

Effects of ADP or AMP-PNP

In Figure 2a,b, 10 mM ADP was present. At both 1:1 (Fig. 2a) and 2:1 (Fig. 2b) $GroES_7$: $GroEL_{14}$, there is a good fit to a single exponential. The rate constants are respectively: 0.20 ± 0.003 s^{-1}, and 0.190 ± 0.003 s^{-1}, with associated amplitudes of 0.0080 ± 0.00004 units and 0.0077 ± 0.00006 units. Values of χ^2 are 7.8×10^{-5} and 8.1×10^{-5}, respectively, compared with 9.8×10^{-5} and 9.5×10^{-5} for fits to double

exponentials. In Figure 2c,d, 15 mM AMP-PNP was used as the nucleotide. At 1:1 $GroES_7$: $GroEL_{14}$ (Fig. 2c), there is a good fit to a single exponential. The rate constant is 0.22 ± 0.004 s^{-1} and amplitude 0.0072 ± 0.00004 units. The value of χ^2 is 9.9×10^{-5}, compared with 9.34×10^{-5} for a fit to a double exponential. At 2:1 $GroES_7$: $GroEL_{14}$ (Fig. 2d), the fit is clearly to a double exponential. The rate constants are 1.72 ± 0.05 s^{-1} and 0.21 ± 0.01 s^{-1}, with respective amplitudes of 0.0045 ± 0.0001 units and 0.0042 ± 0.0001 units; and $\chi^2 = 2.8 \times 10^{-5}$.

Effects of a mixture of ADP and ATP

The experiments with AMP-PNP were repeated using instead a combination of 2.7 mM ATP and 0.38 mM ADP, the approximate concentrations in *E. coli* [20] (data not shown). The single exponential fit for 1:1 $GroES_7$: $GroEL_{14}$ has a rate constant of 0.466 ± 0.007 s^{-1} and amplitude 0.0087 ± 0.00005 units. The double exponential fit for 2:1 $GroES_7$: $GroEL_{14}$ has rate constants of 2.78 ± 0.14 s^{-1} and amplitude 0.004 ± 0.0002 units, and 0.48 ± 0.02 s^{-1} and amplitude 0.0042 ± 0.0002 units.

Folding of barnase on its addition to preformed GroEL·GroES·nucleotide complexes

Denatured barnase was added to a twofold excess of $GroEL_{14}$·$GroES_7$·nucleotide complex (where 'nucleotide' = ATP, its non-hydrolyzable analogue AMP-PNP, or ADP or a mixture of ATP and ADP at physiological concentrations — 2.7 and 0.38 mM, respectively [20]). Barnase folded with a rate constant of 2.4 s^{-1} in the presence of ATP, 1.9 s^{-1} in the presence of ADP, 2.1 s^{-1} in the presence of ATP and ADP, and 1.6 s^{-1} in the presence of AMP-PNP compared with 9.8 s^{-1} for folding in the absence of chaperone. The time course for folding of barnase fitted well to a first-order exponential for all nucleotides (Fig. 3), which did not vary as the ratio of barnase to the GroEL·GroES·nucleotide complex changed. Changing the stoichiometry of $GroES_7$: $GroEL_{14}$ in the mixture from 1:1 to 2:1 did not observably affect the kinetics.

Discussion

Previously [18], a sequence of four phases was observed on the addition of denatured barnase to GroEL in the absence of GroES. First, a fast phase representing the folding of barnase that is free in solution (9.8 s^{-1}), combined with its rate of binding to GroEL (with a stoichiometry of up to 4 mol barnase bound per mole $GroEL_{14}$ at very high concentrations of denatured protein). Second, a conformational change in the complex, the rate constant of which (2 s^{-1}) was not affected by mutations of barnase that alter its rate of folding in solution. Third, the folding of multiply bound moles of barnase at 0.24 s^{-1}. Fourth, the folding of the singly bound mole of barnase at 0.025 s^{-1}. The addition of ATP speeded up the two slow phases by a factor of 15 at saturating concentrations [18]. Under conditions of $GroEL_{14}$: barnase of 2:1, the slow folding of the singly bound mole of barnase dominates [18]. The kinetics of folding in the presence of nucleotides and $GroES_7$ is much simpler: only the initial binding phase is seen and, depending on the order of addition and ratio of $GroES_7$: $GroES_{14}$, either one or two folding phases whose rates are sensitive to mutations (see below). The stoichiometry of denatured barnase bound per mole of $GroEL_{14}$·$GroES_7$ complex reached a maximum of only 3:1 at very high ratios of barnase to GroEL (data not shown). The experiments performed here were at ratios of barnase to $GroEL_{14}$·$GroES_7$ of 1:2 where the distribution of complexes in solution is nearly 50% $GroEL_{14}$·$GroES_7$ and 50% $GroEL_{14}$·$GroES_7$·$barnase_1$, with very little multiply bound complex being present.

Single phase folding kinetics on addition of barnase to the preformed GroEL·GroES·nucleotide complexes

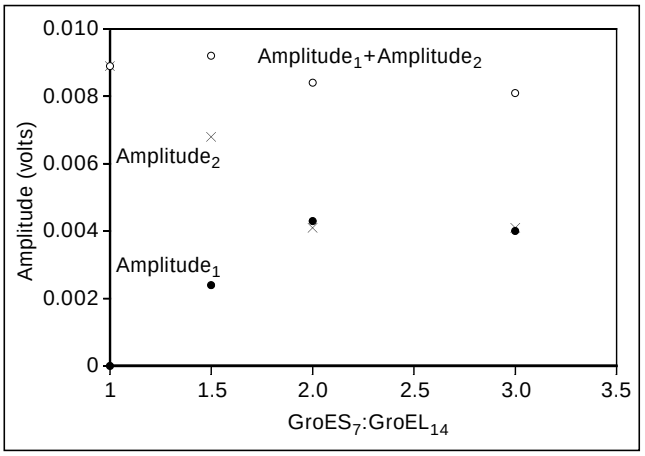
Only simple monophasic folding kinetics are seen on the addition of barnase to preformed GroEL·GroES·nucleotide complexes. The $GroEL_{14}$·$GroES_7$·denatured barnase·nucleotide complexes formed by this route are fast-folding complexes, all folding at about 2 s^{-1}, with small variations depending on the nucleotide used to form the complex and the precise effect of the nucleotide on the conformation of the complex. There is no evidence in the present study of where barnase binds, but it is expected from other studies [9,10] that denatured barnase binds to the ring of GroEL that is *trans* to GroES in the 1:1 $GroES_7$: $GroEL_{14}$ complex.

Biphasic folding kinetics on addition of GroES and ATP or AMP-PNP to the preformed GroEL·denatured barnase complex

The $GroEL_{14}$·$GroES_7$·denatured barnase·nucleotide complexes formed by the alternative route of adding a stoichiometric quantity of $GroES_7$ to the $GroEL_{14}$·nucleotide·denatured barnase complex are slower-folding complexes (Fig. 1a; Fig. 2a–c). By analogy with other studies [9,14], barnase is probably encapsulated in the *cis* ring of GroEL. The folding traces again fitted perfectly to single exponential traces for the nucleotides being ATP, AMP-PNP, ADP or a mixture of ATP and ADP at physiological concentrations (Figs 1,2).

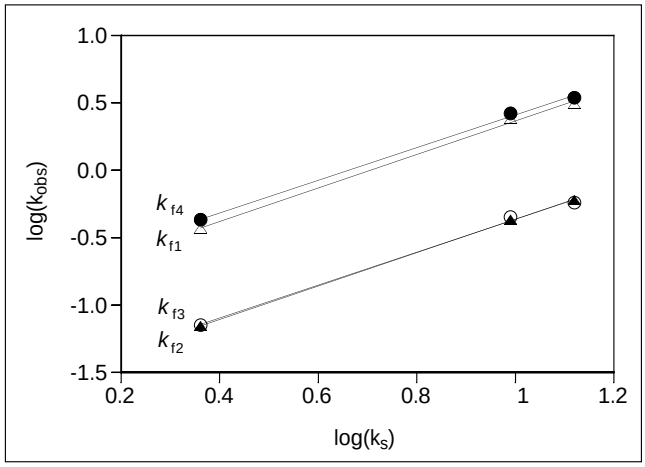
But doubling the concentration of GroES (the nucleotide being ATP, AMP-PNP, or the physiological mixture of ATP and ADP, but not ADP alone) led to the kinetics being biphasic, with the phases having nearly equal amplitudes (Figs 1,2). The amplitude of each signal is 50% of the amplitude of the single rate constant for 1:1 stoichiometry of $GroES_7$: $GroEL_{14}$. A single phase only was found in the presence of ADP when $GroES_7$: $GroEL_{14}$ = 2:1 (Fig. 2a,b). Importantly, footballs do not form in the pres-

Figure 4



Relative amplitudes of phases 1 and 2 on variation of $GroES_7$: $GroEL_{14}$. Conditions as in Fig. 1, the folding of barnase was monitored on the addition of 1 mM ATP and different concentrations of GroES to the preformed GroEL·denatured barnase complex.

Figure 5



Plots of logarithms of rate constants for refolding of mutants of barnase in the presence of GroEL and GroES versus the values in their absence. Plotted versus the logarithm of the rate constant for folding in solution, $\log k_s$ (units of s^{-1}), are the logarithms of k_{f1} for the folding of barnase on its addition to $GroEL_{14}$·$GroES_7$ which has been preformed in the presence of 1 mM ATP; k_{f2} for the folding of barnase on the addition of 1:1 $GroES_7$ to $GroEL_{14}$·denatured barnase, with the same nucleotide; and the two values of k_{f3} and k_{f4} on the addition of 2:1 $GroES_7$ to $GroEL_{14}$·denatured barnase, with the same nucleotide. The equations for the lines are $\log k_{f1} = -0.87 + 1.24 \times \log k_s$; $\log k_{f2} = -1.6 + 1.24 \times \log k_s$; $\log k_{f3} = -1.59 + 1.22 \times \log k_s$; and $\log k_{f4} = -0.8 + 1.21 \times \log k_s$. Very similar correlations were obtained when both ATP and ADP (2.7 and 0.38 mM respectively) were present.

ence of ADP alone [11]. Further ratios of $GroES_7 : GroEL_{14}$ were examined in the presence of ATP (Fig. 1). At 1.5:1, biphasic kinetics were just detectable, and the amplitudes of fast to slow were 1:3. At 3:1 $GroES_7 : GroEL_{14}$, the amplitudes were still 50:50 (Fig. 4). In each case, the rate constant of the fast phase at ratios of $GroES_7 : GroEL_{14}$ ≥2:1 was the same as that found on the addition of denatured barnase to the $GroEL_{14}$·$GroES_7$·nucleotide complex.

Mutants show each phase is a folding event

We can use mutants of barnase to show that each of the rate constants measured is that for the folding of barnase and not that for some conformational change in GroEL that affects the tryptophan spectrum. The mutation Ser91→Ala slows down [21] and the triple mutation Asp8→Ala; Asp12→Ala; Arg110→Ala speeds up folding in solution [22]. The rate constants for all the phases change in parallel with those in solution on mutation of barnase, with slopes close to unity (Fig. 5). Thus, the fluorescence changes reflect folding events.

Implication of a transient $GroEL_{14}$·$(GroES_7)_2$·denatured barnase complex

The additional phase in the kinetics with increasing concentrations of $GroES_7$ from a stoichiometry of 1:1 with $GroEL_{14}$ to 2:1 implies that a $GroEL_{14}$·$(GroES_7)_2$·denatured barnase complex is formed. Further, a single mole of denatured barnase bound to an otherwise symmetrical $GroEL_{14}$·$(GroES_7)_2$ complex would constitute just a single chemical entity and so it would fold as a single species and not by a pair of parallel pathways. There are three possibilities that could explain these observations.

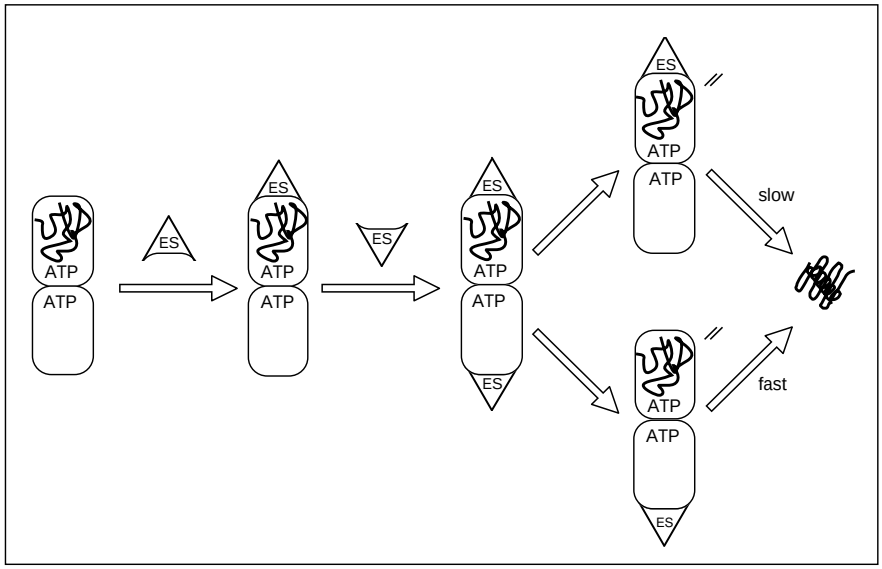
The biphasic kinetics result from sequential reactions

This possibility is unlikely from the results on the folding of mutants. The slopes of close to 1 for the logarithms of rate constants of all the reactions studied in the presence of GroEL against those in solution (Fig. 5) are indicative of parallel folding pathways in the $GroEL_{14}$·$(GroES_7)_2$·denatured barnase complex rather than for sequential steps. It would be very strange if the rate-determining process for folding of a singly bound mole of barnase to $GroEL_{14}$ or to $GroEL_{14}$·$GroES_7$ parallels that in solution and has the same sensitivity to mutation, but the $GroEL_{14}$·$(GroES_7)_2$·denatured barnase complex folds in two successive steps, each of which is as sensitive to mutation as is the overall rate-determining process.

Two moles of barnase are bound cis

It is also unlikely that there are two moles of barnase bound per mole of $GroEL_{14}$·$(GroES_7)_2$·denatured barnase complex under the conditions of excess $GroEL_{14}$·$(GroES_7)_2$: barnase when two equivalents of $GroES_7$ are added to the $GroEL_{14}$·denatured barnase complex. Further, in the absence of GroES, that complex has primarily a single slow folding phase under these conditions [18], and there is only a single phase in the presence of stoichiometric quantities of $GroES_7$.

Figure 6



Scheme for the formation of slow and fast folding complexes. GroES and nucleotides were added to the GroEL·denatured protein complex in our experiments. A GroEL·denatured protein complex is formed transiently during folding *in vivo* from the GroEL·GroES·nucleotide·denatured protein complex on hydrolysis of ATP and expulsion of GroES. GroES binds preferentially to the *cis* GroEL ring. If GroES is not in excess, then slow folding will occur. Excess GroES will rapidly bind to form the transient *pseudo*-symmetric complex, which then splits 50:50 to give *cis* and *trans* forms. ATP hydrolysis is not required for this step as the non-hydolyzable AMP-PNP may be substituted for ATP. The *trans* complex that is formed on the loss of GroES from the football must be in a different conformation from the initial *trans* GroEL$_{14}$·GroES$_7$·nucleotide·denatured protein complex.

Two different GroEL$_{14}$·GroES$_7$·denatured barnase complexes are formed via a transient GroEL$_{14}$·(GroES$_7$)$_2$ complex

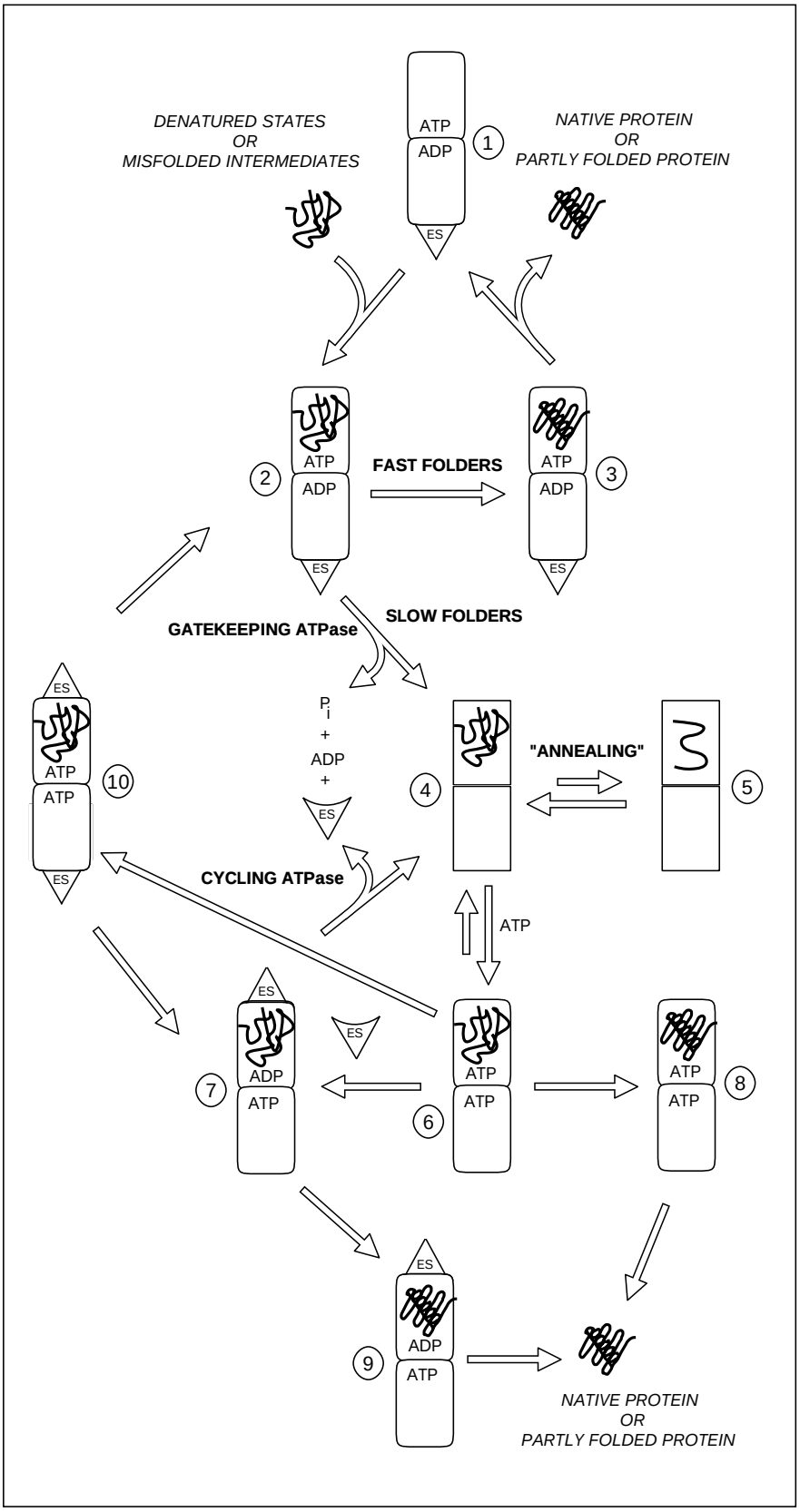
The simplest explanation of the kinetic data is that two different GroEL$_{14}$·GroES$_7$·denatured barnase complexes are formed from a transient GroEL$_{14}$·(GroES$_7$)$_2$·denatured barnase complex. First, GroES binds to just one ring, presumably the *cis* ring from other studies [9,10], and then excess GroES binds to the other, presumably *trans*. Then, one ring of GroES$_7$ dissociates with equal probability from the *cis* and *trans* GroEL rings in the GroEL$_{14}$·(GroES$_7$)$_2$ complex to generate the two different complexes (Fig. 6). The two different GroEL$_{14}$·GroES$_7$·denatured barnase complexes do not equilibrate on a time scale that competes with the subsequent folding of barnase. If they did equilibrate rapidly, then there would be a single rate constant for folding that would be a weighted mean of those from the two states.

The evidence for the scheme in Figure 6 is thus indirect, but it is the only one of the three simple possibilities that can explain the data. Further, it implies that the initial *cis* GroEL$_{14}$·GroES$_7$·denatured barnase complex that is formed is different from the final *cis* complex: the initial *cis* complex has to bind rapidly the second equivalent of GroES to generate 50% each of *cis* and *trans* complexes, but the final one cannot because there appears to be no further equilibration, as argued above.

A biological role for footballs

These observations point to a biological role of footballs in the chaperone cycle of slowly folding proteins. GroEL$_{14}$·protein complexes are transiently formed during the chaperone cycle from GroEL$_{14}$·GroES$_7$·protein complexes as ATP hydrolyzes and expels GroES$_7$. Our kinetic data show that, on rebinding of GroES, there is a very strong preference for the formation of just one GroEL$_{14}$·GroES$_7$·protein complex, which, from other data [9,10], is the *cis* complex in which the protein is fully encapsulated. If the *cis* complex is preferentially formed, then this poses the question of how a protein escapes from the GroEL cage. (Note that barnase folds while bound to GroEL and does not need to dissociate for folding to occur. The binding of native barnase to GroEL has been detected by NMR line broadening [19], and the folding of other small proteins that are trapped in GroEL has been observed [10,23].) On opening of the GroES 'trapdoor' on the hydrolysis of ATP, there is competition between escape of a newly folded protein and the rebinding of GroES. There is ample time for small fully folded proteins to dissociate as they are weakly bound and have high dissociation rate constants. But partly folded proteins that are subunits of larger complexes will have exposed hydrophobic surfaces that bind tightly to GroEL and slow down dissociation. This difficulty will be overcome if there is the occasional binding of GroES to a *cis* GroEL$_{14}$·GroES$_7$·protein complex to give the football that then dissociates to give 50% of a *trans* GroEL$_{14}$·GroES$_7$·protein complex. Slowly dissociating proteins will have time to escape, and subunits of multimeric proteins will have a chance of being exposed to their partners. It must be emphasized that footballs come into this scheme as a transient species and we are not implying that they are the predominant species in solution. Indeed, our experiments are performed under sub-optimal concentrations of $MgCl_2$ for their formation [15]. We would argue that the kinetically important resting state of GroEL is the GroEL$_{14}$·GroES$_7$·ATP complex [16]. The previously postulated mechanism for GroEL can now be modified to include the transient footballs (Fig. 7).

Figure 7



Modification of the 'Folding and Annealing Cage' mechanism [16]. The evidence for this scheme is detailed in reference [16] where it is discussed. The modification here is the addition of species 10 (the football), via which 6 can be shunted back to 2. Briefly, the denatured state of a protein binds to the *trans* ring of GroEL in the resting GroEL·GroES·nucleotide complex 1 in the cell, which is the weak-binding/fast-folding R-state. An inherently fast-folding protein, such as the major fraction of denatured barnase, folds before the slow ATPase activity of GroEL occurs. Slow-folding proteins remain bound for sufficiently long that the ATPase activity occurs, which causes the comcomitant expulsion of GroES to give the tight-binding/slow-folding T-state 4 that has high annealing potential [19]. This ATPase activity is the 'gatekeeper' that selects which species require chaperoning. GroES rebinds preferentially to the *cis* ring of 6 to give 7, in which the denatured protein is encapsulated. The 'cycling' ATPase then converts 7 to 4. There is, thus, a cycling between R-states, that allow fast folding, and the T-state, that can melt out misfolding until the protein becomes sufficiently folded. Periodically, two rings of GroES bind to 6 and form the football 10, which breaks down to give either 7 or 2. A native or partly folded state in 2 can either dissociate or, if part of a multimeric complex, be exposed to its other subunits. Other intermediate states have been omitted from the cartoon for clarity.

Materials and methods

Protein purification

Barnase mutants used in this study were Trp94→Tyr [24], Ser91→Ala; Trp94→Tyr and Asp8→Ala; Asp12→Ala; Trp94→Tyr; Arg110→Ala. They were prepared by inverse PCR, expressed and purified as described [25]. Protein concentrations were calculated from ϵ_{280}= 23 200, according to the method of Gill and von Hippel [26].

GroEL and GroES were overexpressed in the *E. coli* strain TG2 containing the plasmid pOF39. Cells were grown in 2 × TY medium overnight. After harvesting and centrifugation, the cell paste was resuspended in 100 mM Tris/HCl pH 8.1, 10 mM DTT, 0.1 mM EDTA, containing 0.1 mg ml^{-1} DNase I and 0.2 mg ml^{-1} PMSF. After sonication, cell debris was removed by centrifugation and 30 and 80% ammonium sulfate precipitation steps were carried out. The pellet from the last precipitation was resuspended in 50 mM Tris pH 7.2, 2 mM DTT and 0.1 mM EDTA and dialyzed into the same buffer. The dialyzed sample was loaded into a 500 ml DEAE-Sephacel column equilibrated in the same buffer. Elution was performed using a linear gradient from 0–500 mM NaCl. GroES elutes at 0.18 M and GroEL at 0.31 M NaCl. From this point purification of each protein follow different procedures. Fractions containing GroEL were pooled and processed as previously described [17]. GroEL was more than 98% pure. Protein concentration was determined by Bradford assay [27] and confirmed by quantitative amino acid analysis.

GroES fractions were pooled, quickly heated to 58°C and incubated at 58°C for another 30 min. Precipitated material was removed by centrifugation and the soluble protein was dialyzed into Tris 50 mM pH 7.2, and 0.4 M $(NH_4)_2SO_4$. At this stage GroES is 90% pure. Dialyzed solution was chromatographed on a 150 ml Phenyl-Sepharose column equilibrated in the same buffer. Sample elution was performed using a linear gradient from 0.4–0 M $(NH_4)_2SO_4$. GroES eluted at 0.17 M $(NH_4)_2SO_4$. Protein was more than 98% pure at this stage. The protein solution was dialyzed into 5 mM Tris pH 7.2. GroES concentration was determined by Bradford assay [27] and confirmed by quantitative amino acid analysis.

Refolding of barnase

The kinetics of folding of barnase was measured using an Applied Photophysics SX-17MV stopped flow spectrofluorimeter, as described [18]. Additional mixers and delay loops were fitted so that two solutions could be mixed together, allowed to incubate for a predetermined time interval and then mixed with a third. The excitation wavelength was 290 nm (10 nm bandpass) and emission wavelength 315 nm (10 nm bandpass), where the increase in fluorescence on folding is maximal. The mutant barnase Trp94→Tyr (which has enhanced fluorescence characteristics because energy transfer from Trp71 to Trp94 lowers the fluorescence yield of wild-type barnase by a factor of 3 [24]) was denatured in 32 mM HCl (pH 1.5) and mixed with a folding buffer (containing various concentrations of GroEL or GroES and nucleotides, where appropriate) to give final concentrations of 100 mM 2-[*N*-morpholino]ethanesulfonic acid (MES), pH 6.3, 2 mM KCl, 2 mM $MgCl_2$, 1 μM barnase, and generally 2 μM GroEL$_{14}$, at 25°C. The fluorescence was monitored using a logarithmic time base that gives more points at shorter times. Five separate runs were averaged for each concentration of GroES. The time courses were smoothed further by a sliding window of five time points: this does not affect the amplitudes or rate constants analyzed using the program Kaleidagraph™ (Abelbeck software) but lowers high frequency noise.

References

1. Ellis, R.J. & Hartl, F.U. (1996). Protein folding in the cell: competing models of chaperonin function. *FASEB J.* **10**, 20–26.
2. Lorimer, G.H. & Todd, M.J. (1996). GroE structures galore. *Nat. Struct. Biol.* **3**, 116–121.
3. Braig, K., *et al.*, & Sigler, P.B. (1994). The crystal structure of the bacterial chaperonin GroEL at 2.8 Å. *Nature* **371**, 578–586.
4. Braig, K., Adams, P.D. & Brunger, A.T. (1995). Conformational variability in the refined structure of the chaperonin GroEL at 2.8 Å resolution. *Nat. Struct. Biol.* **2**, 1083–1094.
5. Chandrasekhar, G.N., Tilly, K., Woolford, C., Hendrix, R. & Georgopoulos, C. (1986). Purification and properties of the GroES morphogenetic protein of *Escherichia coli*. *J. Biol. Chem.* **261**, 12414–12419.
6. Chen, S., *et al.*, & Saibil, H.R. (1994). Location of a folding protein and shape changes in GroEL–GroES complexes imaged by cryo-electron microscopy. *Nature* **371**, 261–264.
7. Saibil, H. (1996). The lid that shapes the pot: structure and function of the chaperonin GroES. *Structure* **4**, 1–4.
8. Langer, T., Lu, C., Echols, H., Flanagan, J., Hayer, M.K. & Hartl, F.U. (1992). Successive action of DnaK, DnaJ and GroEL along the pathway of chaperone-mediated protein folding. *Nature* **356**, 683–689.
9. Weissman, J.S., *et al.*, & Horwich, A.L. (1995). Mechanism of GroEL action: productive release of polypeptide from a sequestered position under GroES. *Cell* **83**, 577–587.
10. Mayhew, M., Da Silva, A.C.R., Martin, J., Erdjument-Bromage, H., Temps, P. & Hartl, F.U. (1996). Protein folding in the central cavity of the GroEL–GroES chaperonin complex. *Nature* **379**, 420–426.
11. Azem, A., Kessel, M. & Goloubinoff, P. (1994). Characterization of a functional GroEL$_{14}$(GroES$_7$)$_2$ chaperonin hetero-oligomer. *Science* **265**, 653–656.
12. Schmidt, M., *et al.*, & Buchner, J. (1994). Symmetric complexes of GroE chaperonins as part of the functional cycle. *Science* **265**, 657–659.
13. Azem, A., Diamant, S., Kessel, M., Weiss, C. & Goloubinoff, P. (1995). The protein-folding activity of chaperonins correlates with the symmetric GroEL$_{14}$(GroES$_7$)$_2$ heterooligomer. *Proc. Natl. Acad. Sci. USA* **92**, 12021–12025.
14. Llorca, O., Carrascosa, J.L. & Valpuesta, J.M. (1996). Biochemical characterization of symmetric GroEL–GroES complexes. Evidence for a role in protein folding. *J. Biol. Chem.* **271**, 68–76.
15. Engel, A., *et al.*, & Hartl, F.U. (1995). Functional significance of symmetrical versus asymmetrical GroEL–GroES chaperonin complexes. *Science* **269**, 832–836.
16. Corrales, F.J. & Fersht, A.R. (1996). Towards a mechanism for GroEL–ES chaperone activity: an 'ATPase-gated and -pulsed folding and annealing cage'. *Proc. Natl. Acad. Sci. USA*, in press.
17. Gray, T.E. & Fersht, A.R. (1993). Refolding of barnase in the presence of GroEL. *J. Mol. Biol.* **232**, 1197–1207.
18. Corrales, F.J. & Fersht, A.R. (1995). The folding of GroEL-bound barnase as a model for chaperonin-mediated protein folding. *Proc. Natl. Acad. Sci. USA* **92**, 5326–5330.
19. Zahn, R., Perrett, S., Stenberg, G. & Fersht, A.R. (1996). Catalysis of amide proton exchange by the molecular chaperones GroEL and SecB. *Science* **271**, 642–645.
20. Matthews, C.K. (1972). Biochemistry of deoxyribonucleic acid-defective amber mutants of bacteriophage T4. *J. Biol. Chem.* **247**, 7430–7438.
21. Matouschek, A., Serrano, L. & Fersht, A.R. (1992). The folding of an enzyme. IV. Structure of an intermediate in the refolding of barnase analysed by protein engineering procedure. *J. Mol. Biol.* **224**, 819–835.
22. Horovitz, A. & Fersht, A. (1992). Co-operative interactions during protein folding. *J. Mol. Biol.* **224**, 733–740.
23. Weissman, J.S, *et al.*, & Horwich, A.L. (1996). Characterization of the active intermediate of a GroEL–GroES-mediated protein folding reaction. *Cell* **84**, 481–490.
24. Loewenthal, R., Sancho, J. & Fersht, A.R. (1991). Fluorescence spectrum of barnase: contributions of three tryptophan residues and a histidine-related pH-dependence. *Biochemistry* **30**, 6775–6779.
25. Serrano, L., Horovitz, A., Avron, B., Bycroft, M. & Fersht, A.R. (1990). Estimating the contribution of engineered surface electrostatic interactions to protein stability by using double-mutant cycles. *Biochemistry* **29**, 9343–9352.
26. Gill, S.C. & Von Hippel, P.H. (1989). Calculation of protein extinction coefficients from amino acid sequence data. *Analyt. Biochem.* **182**, 319–326.
27. Bradford, M.M. (1976). A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein dye binding. *Analyt. Biochem.* **72**, 248–254.